Transcriptional Map of Respiratory Versatility in the Hyperthermophilic Crenarchaeon *Pyrobaculum aerophilum*^{∇†}

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Hyperthermophilic crenarchaea in the genus *Pyrobaculum* are notable for respiratory versatility, but relatively little is known about the genetics or regulation of crenarchaeal respiratory pathways. We measured global gene expression in *Pyrobaculum aerophilum* cultured with oxygen, nitrate, arsenate and ferric iron as terminal electron acceptors to identify transcriptional patterns that differentiate these pathways. We also compared genome sequences for four closely related species with diverse respiratory characteristics (*Pyrobaculum arsenaticum*, *Pyrobaculum caldifontis*, *Pyrobaculum islandicum*, and *Thermoproteus neutrophilus*) to identify genes associated with different respiratory capabilities. Specific patterns of gene expression in *P. aerophilum* were associated with aerobic respiration, nitrate respiration, arsenate respiration, and anoxia. Functional predictions based on these patterns include separate cytochrome oxidases for aerobic growth and oxygen scavenging, a nitric oxide-responsive transcriptional regulator, a multicopper oxidase involved in denitrification, and an archaeal arsenate respiratory reductase. We were unable to identify specific genes for iron respiration, but *P. aerophilum* exhibited repressive transcriptional responses to iron remarkably similar to those controlled by the ferric uptake regulator in bacteria. Together, these analyses present a genome-scale view of crenarchaeal respiratory flexibility and support a large number of functional and regulatory predictions for further investigation. The complete gene expression data set can be viewed in genomic context with the Archaeal Genome Browser at archaea.ucsc.edu.

With the exception of the euryarchaeal halophiles, many archaeal model organisms are either obligate aerobes (e.g., *Sulfolobus* spp.) or obligate anaerobes (e.g., the *Thermococcales*), with limited respiratory flexibility. As a consequence, relatively little is known about the genetics, regulation, and enzymology of archaeal respiratory pathways compared to those of bacteria. Species in the genus *Pyrobaculum* are notable both for respiratory versatility, exemplified by *Pyrobaculum aerophilum*, and for diversity in respiratory capabilities between closely related species (6, 28, 29, 31, 65), presenting a potential model system for investigating respiratory pathways in the crenarchaea.

Pyrobaculum species grow optimally at 90 to 100°C and are common constituents of microbial communities in neutral pH geothermal springs and shallow marine hydrothermal vents (39, 42, 61). *Pyrobaculum aerophilum* is a facultative chemoautotroph that can use oxygen, nitrate, nitrite, arsenate, selenate, selenite, soluble ferric iron citrate, and insoluble ferric iron oxide as respiratory electron acceptors. The complete genome sequence of *P. aerophilum* was published in 2002 (23). The genome sequences of four additional *Pyrobaculum* species

(*P. arsenaticum*, *P. caldifontis*, *P. islandicum*, and *Thermoproteus neutrophilus* [to be reclassified as a true *Pyrobaculum*]) have recently been completed (T. M. Lowe et al., unpublished data), providing a valuable resource for comparing gene content to respiratory characteristics within a closely related group of crenarchaea (Table 1).

A number of *Pyrobaculum* gene products have been characterized by heterologous expression or purification of native proteins, helping to identify genes for aerobic growth, oxidative stress, and denitrification (1, 4, 5, 18, 27, 44, 66). Although *Pyrobaculum* species are among the best-studied archaeal iron respirers, and are the only known archaeal arsenate respirers, the genes required for archaeal arsenate and iron respiration have not been identified (20, 21, 29, 31).

We used DNA microarrays to compare genome-wide expression patterns in *P. aerophilum* cultures with oxygen, nitrate, arsenate, or ferric iron-citrate as terminal electron acceptors. The results support previous observations related to aerobic respiration and suggest new candidate gene functions and regulatory relationships for denitrification and arsenate respiration. The genes for iron respiration remain enigmatic; however, genes repressed by iron suggest a transcriptionally regulated homeostatic response not previously described in the archaea.

MATERIALS AND METHODS

Culture conditions for *P. aerophilum*. *P. aerophilum* IM2 cultures derived from DSMZ strain 7523 were generously provided by Christopher House, Penn State University. Cultures of *P. aerophilum* were grown in media containing (per liter) 10 g NaCl, 1.3 g (NH₄)₂SO₄, 0.28 g KH₂PO₄, 0.25 g

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TABLE 1. Genomic and respiratory characteristics of selected *Pyrobaculum* species

Species	Genome size (Mbp), gene count, GC %	Oxygen tolerance	Terminal electron acceptors supporting growth	Terminal electron acceptors not supporting growth	Reference(s)
<i>P. aerophilum</i>	2.2, 2,706, 51	Facultative microaerobe	O ₂ , NO ₃ ⁻ , NO ₂ ⁻ , As(V), Se(VI), Se(IV), S ₂ O ₃ ²⁻ , ^a Fe(III)-citrate, FeOOH	S ⁰ , N ₂ O ^b	2, 20, 21, 29, 31, 65
<i>P. arsenaticum</i>	2.1, 2,408, 55	Strict anaerobe	S ⁰ , S ₂ O ₃ ²⁻ , As(V), Se(VI), Fe(III)-citrate, FeOOH	O ₂ , NO ₃ ⁻ , Se(IV)	21, 29
<i>P. calidifontis</i>	2.0, 2,200, 57	Facultative aerobe	O ₂ , NO ₃ ⁻ , As(V), ^d Fe(III)-citrate, FeOOH	NO ₂ ⁻ , ^c S ⁰ , S ₂ O ₃ ²⁻ , SO ₃ ²⁻ , SO ₄ ²⁻	6, 21
<i>P. islandicum</i>	1.8, 2,062, 50	Strict anaerobe	S ⁰ , S ₂ O ₃ ²⁻ , SO ₃ ²⁻ , L-cystine, oxidized glutathione, As(V), ^a Fe(III)-citrate, FeOOH	DL-Lanathionine, fumarate, (CH ₃) ₂ SO ₂ , S ₄ O ₆ ²⁻ , SO ₄ ²⁻	21, 28, 31
<i>T. neutrophilus</i>	1.8, 2,053, 60	Strict anaerobe	S ⁰		22

^a Weak growth, final densities of $\leq 10^7$ cells/ml.

^b *P. aerophilum* reduced NO₃⁻ and NO₂⁻ to N₂ during growth (2, 65) and N₂O reductase activity has been demonstrated in *P. aerophilum* cells (16), but N₂O provided as the sole electron acceptor did not support growth (65).

^c *P. calidifontis* reduced NO₃⁻ to N₂ during growth, but NO₂⁻ as the sole electron acceptor did not support growth (6).

^d See Results and Discussion.

MgSO₄ · 7H₂O, 1 mg NaSeO₄, 0.1 mg Na₂WO₄ · 2H₂O, 70 mg CaCl₂ · 2H₂O, 1 mg (NH₄)₂Fe(SO₄)₂ · 6H₂O, 0.5 mg resazurin, 0.5 g yeast extract, and 10 ml of a trace element solution. The trace element solution (DSM141) was prepared by combining the following components (per liter) in a 78.5 μM solution of nitrolic-acetic acid adjusted to pH 6.5: 3 g MgSO₄ · 7H₂O, 0.5 g MnSO₄ · H₂O, 1 g NaCl, 0.1 g FeSO₄ · 7H₂O, 0.18 g CoSO₄ · 7H₂O, 0.1 g CaCl₂ · 2H₂O, 0.18 g ZnSO₄ · 7H₂O, 0.01 g CuSO₄ · 5H₂O, 0.02 g KAl(SO₄)₂ · 12H₂O, 0.01 g H₃BO₃, 0.01 g Na₂MoO₄ · 2H₂O, 0.025 g NiCl₂ · 6H₂O, and 0.0003 g NaSeO₃ · 5H₂O. The complete medium preparation was adjusted to pH 6.8 and sterilized by autoclaving.

All *P. aerophilum* cultures were incubated at 96°C, typically with 200 ml of medium in 0.5-liter wide-mouth bottles under a gas headspace of N₂ and shaking at 200 rpm. For the aerobic cultures, 5% (vol/vol) of atmospheric air was added to the headspace for a final concentration of approximately 1% O₂. Three replicate respiratory induction experiments were performed in which cultures were grown with a 1% O₂ gas headspace, followed by amendment with different respiratory substrates when the cultures consumed the initially supplied O₂. No reducing agents were used. The depletion of O₂ in cultures was monitored by recording changes in the redox indicator dye resazurin from pink to clear. After the O₂ was depleted, the cultures were cooled to the ambient temperature, diluted with a 25% volume of fresh growth medium, and dispensed in 200-ml volumes to 0.5-liter bottles in an anaerobic chamber. Separate bottles were prepared for collection at each of three time points. The bottles received 1% (vol/vol) additions from anoxic 1 M stocks of NaNO₃, Na₂HAsO₄, or Fe(III)-citrate (pH 6); 5% (vol/vol) of atmospheric air in the gas headspace; or no added electron acceptor in each induction experiment. These were reequilibrated to the growth temperature (96°C), and after 2.5 h (T₁), 4.5 h (T₂), or 7.5 h (T₃), one bottle from each respiratory condition was collected for RNA preparation. The cells were collected for RNA analysis by centrifugation at 10,000 × g for 10 min. The cell pellets were frozen in liquid N₂ and stored at -80°C.

Metabolite analyses. Subsamples for Fe(III) and Fe(II) analysis were collected directly into an equal volume of 1 M HCl and stored at -20°C. Subsamples for the analysis of other metabolites were filtered using 0.2-μm Costar Spin-X filters (Corning) and stored at -20°C. NO₂⁻ was measured spectrophotometrically using a Lachat Instruments Quickchem FIA+ autoanalyzer. The total concentrations of NO₃⁻ plus NO₂⁻ were measured by the prereduction of NO₃⁻ on a cadmium column, and NO₃⁻ concentrations were calculated as the difference between the reduced and untreated samples (67). As(III) and total inorganic As were measured by hydride generation followed by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Perkin Elmer Optima 4300. The selective hydride generation from As(III) was achieved by diluting samples 1:100 in a 1 M citrate-citric acid buffer (pH 5.3) before mixing in equal volumes with a 1% NaBH₄-0.5% NaOH solution to generate hydrides (7). Total inorganic As was measured by diluting the samples 1:100 in a 5% solution of KI in 3.8 M HCl to reduce As(V) to As(III) prior to hydride generation and ICP-OES. Fe(II) was measured using the ferrozine assay, and Fe(III) was measured indi-

rectly as the difference between the levels of Fe(II) in the reduced and untreated samples (46).

Microarray production and hybridization. Microarray probes for the *P. aerophilum* genome were prepared by the PCR amplification of *P. aerophilum* genomic DNA using primers picked by a custom PERL program (T. M. Lowe, unpublished data) utilizing the Primer 3 oligonucleotide selection software (48). Probe features included 2,726 annotated open reading frames (ORFs), 243 potential ORFs (dubbed "alternative ORFs") (S. Fitz-Gibbon, personal communication), and 894 intergenic regions more than 60 nucleotides (nt) long. Probes for the ORFs and alternative ORFs were designed as 250- to 400-nt tags covering the most unique portions of genes and were biased toward the 3' end of transcripts. The probes for intergenic regions were tiled as overlapping segments of no more than 700 nt each. PCR-amplified probes were evaluated by gel electrophoresis. Ninety-three loci (~2% of the total) with PCR probe products of unexpected sizes or multiple bands were flagged and removed from analysis (see Table S1 in the supplemental material). The majority of these flagged features are intergenic regions, repetitive gene families, and alternative ORFs. The probes were suspended in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer and printed in quadruplicate on GAPS II slides (Corning), which were stored in a desiccating chamber until use.

Total RNA was extracted from the frozen cell pellets using a Polytron tissue homogenizer and Tri reagent (Sigma-Aldrich). RNA samples were treated with DNase I (Ambion) to remove any residual DNA, reextracted with Tri reagent, and normalized to 1 μg/μl before proceeding with the preparation of the cDNA for microarray analysis. Microarray analyses were performed for all 45 RNA samples generated in the three replicate induction experiments, using a pool of all samples as a universal reference for all hybridizations. Aminoallyl-labeled cDNAs were prepared from 5 μg of sample RNA using 5 μg of random hexamers (3 μg/μl) and the Superscript III labeling kit (Invitrogen). These were subsequently labeled with Cy3-*N*-hydroxysuccinimide ester or Cy5-*N*-hydroxysuccinimide ester (GE Healthcare). Spotted microarray slides were hydrated for 3 min in a humidified chamber, snap-dried at 100°C, covalently linked with 250 mJ in a UV cross-linker (Stratagene), and incubated at 42°C in a solution of 5× SSC, 1% bovine serum albumin, and 0.1% sodium dodecyl sulfate (SDS) for 45 min prior to hybridization. For each hybridization, Cy3-labeled cDNA derived from 5 μg of experimental RNA and Cy5-labeled cDNA derived from 5 μg reference pool RNA were combined with 1 mg/ml herring sperm DNA, 0.38 mg/ml yeast tRNA, 4× SSC, and 0.5% SDS in a total volume of 55 μl and hybridized for 14 to 18 h at 60°C in sealed hybridization chambers. After hybridization, the slides were washed for 2 min each in 0.05% SDS plus 0.1× SSC and in 0.05× SSC and dried by centrifugation. The slides were scanned at a 5-μm resolution immediately after washing using a Genepix 4000B microarray scanner (Molecular Devices).

Microarray data analysis. Microarray features were analyzed using Genepix 6 software (Molecular Devices). The median pixel intensities for each spotted feature were normalized using the LOESS function of the open-source Biocon-

ductor package “marray,” so that the normalized global and print-tip median $\log_2(\text{Ch1/Ch2})$ ratio of each array was zero (25). For each individual array feature, the median intensity values of four replicate spots were used for subsequent analyses. An intra-experimental normalization was performed before statistical and clustering analyses by subtracting the average $\log_2$ ratio at each locus within the experiment from the $\log_2$ ratio representing each condition and time point. A statistical analysis of differential expression was performed using Edge software and the SAM package for *R* (57, 58, 63). A false discovery rate (FDR) of 3% from the Edge analysis was used as an upper limit for calling differentially expressed genes statistically significant. The clustering of gene expression profiles and the production of heat maps were performed using Genesis software (59). Normalized $\log_2$ ratios for all loci represented in the microarrays and the results of the statistical analyses are compiled in Table S2A, S2B, and S2C in the supplemental material.

Northern analysis. RNA samples from each of the three replicate respiratory induction experiments were combined in equal amounts to generate 15 samples representing each condition and time point. Ten micrograms of total RNA from each of these samples was denatured for 45 min at 50°C with glyoxal loading buffer (Ambion) and resolved by electrophoresis in 1% agarose gels buffered with 30 mM bis-Tris, 10 mM PIPES, 1 mM EDTA (pH 6.5) (51). Size-separated RNAs were blotted onto Hybond N+ nylon membranes (GE healthcare) using capillary transfer. The PCR products and primers identical to those used to generate microarray probes were used for single-stranded DNA probe preparation. Single-stranded DNA probes were prepared by linear PCR with Strip-EZ PCR reagents (Ambion) and body labeled using [α - ^{32}P]ATP. Hybridizations were carried out at 42°C using Ultrahyb buffer (Ambion). The membranes were stripped of probes and prepared for rehybridization using Strip EZ PCR reagents and protocols.

Computational genomics. Gene features and organization were evaluated using the Archaeal Genome Browser (53). Orthology was inferred by identifying reciprocal best BlastP hits (3) in which protein pairs were required to align at least 85% of full length and have at least 45% amino acid identity. Predicted orthologs for *P. aerophilum* genes in the other sequenced *Pyrobaculum* genomes are listed in Table S3 in the supplemental material. Putative twin-arginine translocation signals were identified using TatP (9). The promoter regions of coregulated genes were screened for possible regulatory motifs using BioProspector (32). The *P. aerophilum* genome was screened for additional occurrences of identified motifs using position-specific scoring matrices. Basal regulatory elements and TATA boxes were identified using position-specific scoring matrices (P. P. Chan and T. M. Lowe, unpublished data). Motif logo figures were generated using Weblogo (13).

Culture conditions for *P. arsenaticum* and *P. calidifontis*. *P. arsenaticum* strain DSM 13514 was grown at 95°C in DSM390 medium reduced with 0.01% Na_2S and maintained with 20 mM $\text{Na}_2\text{S}_2\text{O}_3$ as the terminal electron acceptor. Ten millimolar NaNO_2 and 10 mM NaNO_3 were each tested separately as alternative terminal electron acceptors.

P. calidifontis strain JCM 11548 was grown at 95°C in TY medium (10 g tryptone, 2 g yeast extract per liter) without $\text{Na}_2\text{S}_2\text{O}_3$ and maintained with 10 mM NaNO_3 as a terminal electron acceptor. 10 mM Na_2HAsO_4 was tested as an alternative terminal electron acceptor. Solid medium was prepared using 1.2% Gelrite and 10 mM MgSO_4 as solidifying agents.

Sequence analysis of the *P. calidifontis* Pcal_1601 locus. *P. calidifontis* cells were lysed using SNET lysis buffer, and genomic DNA was extracted using the phenol:chloroform:isoamyl alcohol method (51). Genomic DNA was treated with RNase A (Sigma) and reextracted before analysis. A 1-kb segment of the Pcal_1601 locus (chromosomal position 1489399 to 1490424) was PCR amplified using the forward primer 5'-GAGAGCTACACAGATCTTCG, the reverse primer 5'-GATAAGCACGAAGTCTCAGG, and Phusion high fidelity polymerase (Finnzymes).

Microarray data accession numbers. A description of the microarray platform has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database under the accession number GPL6755. The microarray data produced from this study are deposited in the NCBI GEO database under the accession number GSE11366.

RESULTS AND DISCUSSION

Initial microarray experiments comparing the gene expression patterns of *P. aerophilum* cultured to similar cell densities with nitrate, arsenate, or ferric citrate as the terminal electron acceptor showed differences in gene expression of twofold or

greater at approximately one-third of all loci (data not shown). We observed that the growth rates and maximum densities varied with different terminal electron acceptors, which is consistent with other reports (20, 21), and attributed these global changes in gene expression to differences in growth phase as well as responses related to respiratory metabolism.

To focus on genes that are specifically affected by changes in terminal electron acceptors, we analyzed gene expression in five parallel time courses with cultures shifted from aerobic growth to nitrate, arsenate, ferric iron-citrate, or additional O_2 . A group that received no additional terminal electron acceptor was also included. Gene expression and substrate usage were measured at three time points (2.5, 4.5, and 7.5 h). This interval was designed to allow sufficient time for changes in respiratory substrate usage while minimizing differences in growth phase between the treatment groups.

Substrate usage and growth. Changes in medium oxygenation, monitored using the redox indicator dye resazurin, showed that cultures that received no additional electron acceptor and those amended with 1% O_2 depleted the available oxygen at different stages during the time course. Cultures that received no terminal electron acceptor became anoxic between T_1 and T_2 . Cultures amended with 1% O_2 became anoxic between T_2 and T_3 .

Analysis of the culture medium showed that cultures amended with nitrate, arsenate, or ferric citrate reduced each of these substrates during the time course. Cultures shifted to NO_3^- produced approximately 1 mM NO_2^- by T_3 , measured spectrophotometrically (see Fig. S1A and S1B in the supplemental material). Decreases in the total concentration of NO_3^- plus NO_2^- varied from 0 mM to 0.5 mM between replicate induction experiments, suggesting a low and variable reduction of NO_2^- to gaseous NO , N_2O , or N_2 (see Fig. S1A in the supplemental material). Concentrations of NO_3^- and NO_2^- in the cultures that were induced with O_2 , As(V), Fe(III)-citrate, or no electron acceptor were below 0.04 mM, and no significant changes were detected over the time course (data not shown). Cultures shifted to As(V) reduced approximately 1 mM As(V) to As(III) by T_3 , as measured by ICP-OES (see Fig. S1C and S1D in the supplemental material). Cultures shifted to Fe(III)-citrate produced approximately 1 mM Fe(II) by T_2 and 3.5 mM Fe(II) by T_3 , as measured by the ferrozine assay (see Fig. S1E in the supplemental material).

With the exception of that of the Fe(III)-treated group, the optical densities of all the treatment groups varied by less than 20% by the end of the time course (see Fig. S1F in the supplemental material). The optical densities of the cultures amended with O_2 or NO_3^- increased more than those amended with As(V) or those that received no additional electron acceptor. The growth medium of the Fe(III)-treated cultures darkened substantially over the time course compared to the uninoculated Fe(III)-amended medium, confounding the use of optical absorbance as a proxy for cell density in this group. However, the total RNA yields and relative intensities for 16S and 23S rRNA bands were similar for cultures shifted to Fe(III) and those shifted to As(V), suggesting similar growth for these two groups (data not shown).

Identification of differentially expressed genes. Statistical analysis using Edge identified 543 features (14% of the total) with significant differences in expression (FDR of <3%) be-

tween respiratory treatments. The hierarchical clustering of expression profiles from this group revealed eight major coexpressed clusters that are the focus of subsequent sections. Edge also identified 1,013 loci (~27% of the total) with significant (FDR of <1%) changes in gene expression over the time course, irrespective of respiratory treatment, suggesting that changes in growth phase between sampling time points affected gene expression at a large proportion of loci. The majority of the most dynamic loci were highly ranked in the respiratory response group (median 9.3-fold range for the top 50 respiratory response loci compared to 3.4-fold for the top 50 temporal response genes). The gene expression profiles for the complete set of loci in each group and the genome-wide data set (see File S1 in the supplemental material) can be viewed interactively using the free Genesis software (http://genome.tugraz.at/genesisclient/genesisclient_description.shtml) (59).

The ranked lists of differentially expressed loci reported by SAM were highly correlated with the lists reported by Edge ($R^2 = 0.92$ and $R^2 = 0.98$ for respiratory treatment and time course analyses, respectively). The results from statistical analyses and normalized $\log_2$ expression ratios from microarray analyses are compiled in Table S2A, S2B, and S2C in the supplemental material. All expression data and views of key respiration loci are also readily accessible in the Archaeal Genome Browser at archaea.ucsc.edu (53).

Opposing regulation of aerobic cytochrome oxidases. *P. aerophilum* grows microaerobically under gas headspace O_2 concentrations up to 5% (vol/vol) (65). The *P. aerophilum* genome contains two closely spaced clusters of genes for aerobic cytochrome oxidase complexes (Fig. 1A), described hereafter using Sox nomenclature originating from studies of another crenarchaeal aerobe, *Sulfolobus acidocaldarius* (33, 34). One group of cytochrome oxidase genes encodes components of a SoxB-type enzyme (PAE1355 and PAE1357). Conserved domains in a flanking set of genes (PAE1358-PAE1362) suggest that these may be involved in the biosynthesis of the SoxB complex. A second group of cytochrome oxidase genes (PAE1337-PAE1340) encodes a SoxM-type complex. *Pyrobaculum oguniense* and *Pyrobaculum calidifontis* are both capable of growth under atmospheric O_2 concentrations, and each have syntenic clusters of genes orthologous to the cytochrome oxidase complexes found in *P. aerophilum* (Table 2) (6, 49). The SoxB complex, SoxM complex, superoxide dismutase, and catalase proteins of *P. aerophilum* show strong similarity ($\geq 85\%$, $\geq 67\%$, 88%, and 88% identity, respectively) to those of *P. oguniense* and *P. calidifontis*.

Subunits I (PAE1357) and II (PAE1355) of the *P. aerophilum* SoxB-type cytochrome oxidase complex were upregulated under all conditions in the respiratory time course experiment except the first two time points after amendment with O_2 and all three time points after amendment with nitrate. These oxygen- and nitrate-amended samples showed expression levels up to 15-fold lower (Fig. 1B and D). A small alternative ORF upstream of PAE1355 (PAE1353) and directly downstream of a strong promoter signal showed an identical expression pattern, suggesting that this may represent a small, cotranscribed gene. Northern analysis of PAE1355 showed a band at approximately 600 nt, consistent with the cotranscription of PAE1353 (144 nt) and PAE1355 (477 nt) (Fig. 1D), and a minor band at 2.3 kb (not shown), consistent with the

cotranscription of these genes with PAE1357 (1,674 nt). Downstream genes, including a *senC* homolog (PAE1358) and several hypothetical proteins (PAE1359, PAE1360, and PAE1369), were expressed in a similar pattern (Fig. 1B).

The second group of cytochrome oxidase genes was expressed in a pattern that was essentially the opposite of the PAE1353 to -1357 SoxB-type cytochrome oxidase (Fig. 1C). These genes, annotated as a possible respiratory chain protein (PAE1336), a SoxM-type fusion of cytochrome oxidase subunits I and III (PAE1337), a cytochrome oxidase subunit II (PAE1338), and a SoxI homolog (PAE1340) were upregulated up to 11-fold in oxic (the first two time points following induction with oxygen) versus anoxic samples and upregulated up to 16-fold after induction with nitrate (Fig. 1C, E, and F). Genes encoding subunits of a pyruvate dehydrogenase complex (PAE2644, PAE2646, PAE2648, and PAE2649), as well as an adjacent hypothetical protein with a putative DNA binding domain (PAE2655), were upregulated in a very similar pattern, suggesting enhanced pyruvate consumption associated with the expression of the SoxM cluster of genes (Fig. 1C).

The differential expression of the SoxM-type I/III fusion protein (PAE1337) was not detected in the microarrays, but the coexpression of adjacent genes PAE1336, PAE1338, and PAE1340 prompted us to verify the expression pattern for PAE1337. Northern analyses of PAE1338 and PAE1337 (Fig. 1E and F) showed identical banding patterns that agree with the microarray results for PAE1338, and a major band at approximately 3.3 kb, consistent with the cotranscription of a polycistronic mRNA for PAE1336, PAE1337, PAE1338, and PAE1340 (annotated sizes of 195, 2,403, 450, and 240 nt, respectively). Northern analyses of 20 other key loci showed very good agreement with the expression patterns indicated by the microarray results (Fig. 1 and 2; see also Fig. S2 to S5 in the supplemental material).

Many facultatively aerobic bacteria possess more than one respiratory oxygen reductase for growth under a range of oxygen tensions (47). The regulation of two cytochrome oxidases in the facultative aerobe *Pyrobaculum oguniense* (45) suggests an analogous specialization in the *Pyrobaculum* genus: the *P. oguniense* SoxM-type oxidase was induced under aerobic conditions, whereas the SoxB-type oxidase was induced under anaerobic, thiosulfate-respiring conditions (45). This suggested that the terminal reductase for aerobic growth is the SoxM complex and that the SoxB complex functions in oxygen scavenging with a possible regulatory role.

The opposing expression pattern of the two homologous cytochrome oxidase complexes in *P. aerophilum* corroborates the expression patterns observed in *P. oguniense* and provides support for the distinct functions proposed by Nunoura and colleagues (45). In the current study, *P. aerophilum* SoxB complex genes were upregulated under suboxic conditions, consistent with encoding an oxygen scavenger or sensor (Fig. 1B and D). The *P. aerophilum* SoxM complex genes were expressed in a strikingly opposite pattern, consistent with their primary role in aerobic respiration (Fig. 1C, E, and F). Because *P. oguniense* can grow under atmospheric oxygen concentrations, whereas *P. aerophilum* is a microaerobe, Nunoura et al. speculated that the SoxB complex might be the primary terminal reductase for the microaerobic growth of *P. aerophilum*. Instead, our results

FIG. 1. Genomic organization (A) and transcriptional expression (B to F) of aerobic SoxM-type and SoxB-type cytochrome oxidase genes in *P. aerophilum*. The red arrows in panel A indicate transcripts detected by Northern analysis (D to F). Individual genes are shown in shaded boxes, with arrow marks indicating the predicted direction of transcription. The black hatch marks at the bottom of panel A indicate regions of nucleotide sequence similarity in multiple genome alignments with other *Pyrobaculum* species and show an extended region of similarity in the aerobic *P. calidifontis*. Hierarchically clustered microarray gene expression profiles for SoxB cytochrome oxidase genes (B) and for SoxM cytochrome oxidase genes (C) during time courses of growth on different terminal electron acceptors are shown (No Add, no terminal electron acceptor added). Positive (red) and negative (green) changes in gene expression are shown on a log₂ scale, with the scale bar shown at the top. Expression values for each time course represent the average log₂ ratio of three replicate respiratory induction experiments. The blue dendrograms show relationships based on complete linkage clustering using the Pearson correlation. Northern analyses of the SoxB-type cytochrome oxidase (D) and the SoxM-type cytochrome oxidase genes (E and F), with lanes corresponding to sample growth conditions indicated above the heat maps, are shown. (G) Methylene blue stain of total RNA showing 23S and 16S rRNA bands on a representative blot used for Northern analysis.

indicate that the SoxM-type complex is the terminal reductase for *P. aerophilum* under microaerobic conditions. Given this conclusion, the upregulation of the SoxM complex after induction with nitrate was unexpected. Aerobic cytochrome oxidases are structurally and evolutionarily related

to nitric oxide reductases, and cytochrome oxidases from several bacteria have been shown to reduce nitric oxide (24, 26, 52, 64). However, a direct role for this SoxM-type oxidase in nitrate respiration or denitrification would be novel. Whether the expression of SoxM complex genes is characteristic of

TABLE 2. Proposed functions and predicted orthologs for differentially expressed *P. aerophilum* genes

Locus (annotation)	Proposed function	Conserved domain ^a	Predicted ortholog in <i>Pyrobaculum</i> species			
			<i>P. arsenaticum</i>	<i>P. calidifontis</i>	<i>P. islandicum</i>	<i>T. neutrophilus</i>
PAE1353 (alternative ORF)	O ₂ -scavenging oxidoreductase			Region 5' of Pcal_1950		
PAE1355 (cytochrome <i>c</i> oxidase subunit II)	O ₂ -scavenging oxidoreductase	cl08244: cytochrome <i>c</i> oxidase subunit II		Pcal_1950		
PAE1357 (cytochrome <i>c</i> oxidase subunit I)	O ₂ -scavenging oxidoreductase	cd01660: <i>ba</i> ₃ -like heme-copper oxidase subunit I		Pcal_1949		
PAE1336 (possible respiratory chain protein)	Aerobic O ₂ reductase		Pars_0585			
PAE1337 (cytochrome <i>c</i> oxidase subunit I/III)	Aerobic O ₂ reductase	cl00275: heme-copper oxidase subunit I; cd00386: heme-copper oxidase subunit III		Pcal_1943		
PAE1338 (cytochrome <i>c</i> oxidase subunit II)	Aerobic O ₂ reductase	cl08244: cytochrome <i>c</i> oxidase subunit II		Pcal_1944		
PAE1340 (respiratory chain protein [SoxI homolog])	Aerobic O ₂ reductase			Pcal_1945		
PAE2679 (transcriptional regulator)	NO-responsive transcriptional regulator	cl00088: HTH ARSR superfamily	Pars_0504			
PAE2680 (hypothetical protein)	NO-responsive transcriptional regulator	COG3945: uncharacterized conserved protein	Pars_0505	Pcal_1917		
PAE1888 (multicopper oxidase)	NO ₂ ⁻ or N ₂ O reductase	cl06664: multicopper oxidase				
PAE1263 (molybdopterine oxidoreductase, iron-sulfur-binding subunit)	Arsenate respiratory reductase	COG0437: HybA, Fe-S-cluster-containing hydrogenase	Pars_0387	Pcal_1599	Pisl_1986	Tne_0510
PAE1264 (hypothetical membrane protein)	Arsenate respiratory reductase	cl01295: polysulfide reductase integral transmembrane protein NrfD	Pars_0388	Pcal_1600		
PAE1265 (molybdopterine oxidoreductase, molybdopterine-binding subunit)	Arsenate respiratory reductase	cd02758: MopB tetrathionate reductase; cd02780: molybdopterine-binding, C-terminal (MopB_CT) region of tetrathionate reductase (TtrA); respiratory arsenate As(V) reductase (ArrA)	Pars_0389	Pcal_1601 ^b		
PAE2859 (molybdopterine oxidoreductase, molybdopterine-binding subunit)	Thiosulfate or polysulfide reductase	cd02755: MopB thiosulfate-R-like; cd02775: molybdopterine-binding, C-terminal (MopB_CT)	Pars_0938	Pcal_1500	Pisl_0162	Tne_1951
PAE2860 (molybdopterine oxidoreductase, iron-sulfur subunit)	Thiosulfate or polysulfide reductase	COG0437: HybA, Fe-S-cluster-containing hydrogenase	Pars_0937	Pcal_1499	Pisl_0161	Tne_1952
PAE2861 (molybdopterine oxidoreductase, membrane subunit)	Thiosulfate or polysulfide reductase	cl01295: polysulfide reductase integral transmembrane protein NrfD	Pars_0936	Pcal_1498		
PAE2696 (Mn catalase)	Iron-responsive regulon	cd01051: Mn catalase	Pars_1142	Pcal_0729		
PAE2697 (putative ABC transport protein)	Iron-responsive regulon	cl00427: binding protein-dependent transport system inner membrane component				
PAE2699 [putative Fe(III) ABC transporter ATP-binding protein]	Iron-responsive regulon	cd03255: ABC_MJ0796_Lo1CDE_FtsE				
PAE2700 (putative ABC transport protein)	Iron-responsive regulon	cl02460: transferrin; cl00115: periplasmic binding protein (PBp)				
PAE0600 (hypothetical protein)	Iron-responsive regulon	cl00437: ZIP zinc transporter	Pars_1201	Pcal_1350		

^a From the Conserved Domain Database (CDD). The CDD identifier and product are given. HTH, helix-turn-helix; ARSR, arsenical resistance operon repressor.

^b See Results and Discussion.

steady-state growth on nitrate or is a transitional effect induced by the shift from oxygen to nitrate remains to be determined.

Genes for nitrate respiration and denitrification in *Pyrobaculum*. *P. aerophilum* can reduce nitrate to dinitrogen during growth, and reductase activities for each individual substrate (nitrate [NO₃⁻], nitrite [NO₂⁻], nitric oxide [NO], and

nitrous oxide [N₂O]) in this stepwise reaction have been demonstrated in association with *P. aerophilum* cell membranes (2, 17, 65). The *P. aerophilum* genome contains genes for a *narG*-type nitrate reductase (PAE3611-PAE3613), a *cd*₁-type nitrite reductase (PAE3598 and PAE3600), and a heme O-containing nitric oxide reductase (PAE3603) (1, 18, 23) (see Fig. S2A in

distinct redox optimum for growth on nitrate (20, 21). These results suggest that steady-state growth under controlled redox conditions may produce larger differences in nitrate reductase expression.

Coexpression of denitrification genes. Denitrification genes (PAE3600, the cytochrome d_1 subunit of the cd_1 -type nitrite reductase; PAE3602, a hypothetical protein; PAE3603, the nitric oxide reductase) were upregulated up to 15-fold by the end of the time course after induction with nitrate, compared to aerobic cultures (see Fig. S2C in the supplemental material). A putative transcriptional regulator with homology to MarR-family transcriptional regulators (PAE2679) and an adjacent hypothetical protein with two hemerythrin binding domains (PAE2680) were also strongly upregulated (up to 17-fold) late in the time course after induction with nitrate (see Fig. S2C in the supplemental material). Two other flanking genes (PAE3598, the cytochrome c subunit of the cd_1 -type nitrite reductase; PAE3601, uroporphyrin-III C-methyltransferase, possibly involved in cytochrome heme synthesis) showed similar expression patterns but were less strongly upregulated.

Northern analysis of PAE3600 showed a major band at 1.6 to 1.7 kb and a minor band at 2.2 kb, consistent with a monocistronic transcript of PAE3600 and cotranscription with PAE3601, respectively (see Fig. S2E in the supplemental material). PAE3598 and PAE3603 showed similar expression patterns (see Fig. S2F and S2G in the supplemental material). Northern analysis of the putative MarR-like transcriptional regulator PAE2679 showed a primary band at approximately 1.1 kb, consistent with the cotranscription of PAE2679-PAE2680 (predicted sizes of 526 nt and 543 nt, respectively), and a pattern of expression that resembles those of the nitrite and nitric oxide reductases (see Fig. S2H in the supplemental material). The results of the microarrays and Northern analyses are consistent in showing strong upregulation of these genes at the third time point during growth on nitrate and more moderate upregulation during growth on arsenate (see Fig. S2B to S2H in the supplemental material).

Conserved promoter motif for denitrification genes. An inspection of the PAE3598-PAE3603 locus revealed a conserved promoter motif that may be involved in regulating the transcription of these genes. The motif is palindromic (see Fig. S2I in the supplemental material), which is typical for targets of helix-turn-helix transcription factors, and occurs between the cytochrome c and d_1 components of the nitrite reductase (PAE3598 and PAE3600), in the promoter region of the nitric oxide reductase (PAE3603), and in the promoter region of a hypothetical protein (PAE3602) (see Fig. S2A in the supplemental material). The palindromic halves of the motif show particularly strong conservation between the two nitrite reductase genes, which are divergently transcribed in each of the three *Pyrobaculum* genomes where they occur (see Fig. S2J in the supplemental material).

A candidate nitric oxide-responsive transcriptional regulator. The coexpression of genes for putative MarR-like and hemerythrin-binding proteins (PAE2679 and PAE2680) and genes for denitrification (see Fig. S2C and S2E to S2H in the supplemental material) suggests that these may be regulated by common factors. The upregulation of these genes when nitrite accumulated to ~ 1 mM in the culture medium (see Fig. S1B in the supplemental material) suggests a possible role for

nitrite in their regulation, with relatively low sensitivity to concentrations less than 1 mM. An alternative possible effector is nitric oxide. Bacterial transcriptional regulators of denitrification generally fall in the Crp-Fnr family, are responsive to both oxygen and nitric oxide, and can be sensitive to nanomolar concentrations of nitric oxide (69). In contrast, bacterial MarR family transcriptional regulators typically regulate antibiotic resistance, virulence, and oxidative stress (60). Hemerythrins are di-iron-containing proteins that can reversibly bind oxygen or nitric oxide (43). These annotations suggest that the PAE2679-PAE2680 operon encodes a transcriptional regulator that controls either stress responses or denitrification genes in response to nitric oxide. *P. calidifontis* has an ortholog for only the hemerythrin-like PAE2860, located within a region of the genome that contains the complete suite of genes for denitrification, while *P. arsenaticum* has orthologs for both PAE2679 and PAE2680 (Table 2), flanked on either side by genes for nitrate reductase and nitric oxide reductase.

Upregulation of a multicopper oxidase during nitrate respiration. A putative multicopper oxidase (PAE1888) was among the genes most specifically upregulated (up to fivefold) by growth on nitrate and was highly ranked by statistical analyses (see Fig. S3A and Table S2C in the supplemental material). Northern analysis with a probe for PAE1888 showed a band consistent with the annotated size of 1,434 nt, as well as increasing expression during the time course on nitrate (see Fig. S3B in the supplemental material). The putative 53-kDa enzyme encoded by PAE1888 contains a predicted N-terminal twin-arginine translocation signal, a predicted binuclear Cu-3-type center similar to those in Cu-containing nitrite reductases, and a predicted mononuclear C-terminal Cu-2-type center (36). The twin-arginine signal suggests that this enzyme is exported after assembly to the outer membrane. No orthologs of this gene were found in the other *Pyrobaculum* or *Thermoproteales* genomes (Table 2). The closest BlastP hits ($\sim 40\%$ identity) were annotated as bilirubin oxidases or multicopper oxidases and were from bacteria that are notable for thermophily (*Thermus*, *Aquifex*), catabolism of complex organic substrates (*Polaromonas naphthalenivorans*, “*Candidatus Desulfococcus oleovorans*”), or for ammonia oxidation and denitrification (*Nitrosomonas*, *Nitrosospora*).

Computational scans identified an overrepresented sequence motif (see Fig. S3C in the supplemental material) that may coregulate PAE1888, a hypothetical protein with a thioredoxin-like domain (PAE2750), and two downstream genes (encoding a putative cytochrome c -type biogenesis protein [PAE2751] and a potential noncoding RNA within intergenic region PAE.i1473) that showed similar expression patterns (see Fig. S3A in the supplemental material). The motif is located immediately upstream from the predicted TATA box for both PAE1888 and PAE2750 and is conserved in the promoter regions of genes orthologous to PAE2750 in the genomes *P. calidifontis* and *P. arsenaticum* (see Fig. S3D to S3E in the supplemental material). A putative cytoplasmic siroheme-type ferredoxin-nitrite reductase (PAE2577), typically involved in the assimilatory or detoxifying reduction of nitrite to ammonium (41, 54), also showed an expression pattern similar to PAE1888 (see Fig. S3A in the supplemental material) but lacked this promoter motif.

Enzymes in the multicopper oxidase superfamily are diverse

and typically use oxygen as an electron acceptor but also include bacterial copper-containing nitrite reductases encoded by *nirK* genes and nitrous oxide reductases encoded by *nosZ* genes (70). Based on the specific upregulation of PAE1888 during nitrate respiration and the putative domain structure of the encoded protein, we propose that PAE1888 encodes a novel nitrite or nitrous oxide reductase. The similarity between PAE1888 and genes in *Nitrosomonas* species, which also reduce nitrous oxide and lack identified nitrous oxide reductase genes, provides potential support for a role in nitrous oxide reduction (56, 70). The coregulated, putative thioredoxin (PAE2750) and cytochrome *c* biosynthesis (PAE2751) genes may function in the biosynthesis of cofactors or electron donors for this enzyme.

A predicted ARR. Bacterial arsenate respiratory reductases (ARRs) are members of the molybdopterin oxidoreductase superfamily (50). Degenerate PCR primers that reliably amplify bacterial ARR genes do not detect them in *P. aerophilum* or *P. arsenaticum*, the only archaeal arsenate respirers identified to date (29, 35). We identified nine putative molybdopterin oxidoreductase genes in the *P. aerophilum* genome, four of which (PAE1265, PAE2002, PAE2839/PAE2840, and PAE2859) show limited similarity to functionally characterized genes. We predicted that one of these encodes an ARR similar to those found in bacteria.

BlastP searches of the *P. aerophilum* genome using the Mo-containing subunit of ARR from diverse bacteria (*Shewanella* sp. strain ANA-3, *Bacillus arseniciselenatis*, *Chrysiogenes arsenatis*, *Sulfurospirillum barnesii*) as query sequences all returned PAE2859 as the strongest match. However, the PAE2859 operon was upregulated under anoxic conditions only in the absence of nitrate, arsenate, or ferric iron (Fig. 2B and E). In contrast, a group of genes encoding a putative 132-kDa molybdopterin oxidoreductase (PAE1265), a putative 23-kDa iron-sulfur-protein (PAE1263), and a possible 40.7-kDa membrane-anchoring subunit (PAE1264) were strongly upregulated (up to eightfold) in cultures induced with arsenate (Fig. 2A). These cultures reduced approximately 1 mM arsenate to arsenite (see Fig. S1C and S1D in the supplemental material). Northern analysis with probes for the Mo-containing subunit PAE1265 and the membrane subunit PAE1264 showed transcripts of approximately 5.2 kb and identical patterns of upregulation, consistent with the transcription of an operon containing all three of these coregulated genes (Fig. 2C and D). Northern analysis also showed increasing expression in cultures induced with ferric iron and upregulation at the last time point after induction with nitrate.

The predicted active site domain of PAE1265 is conserved in bacterial tetrathionate reductases and ARRs (36). Both the iron-sulfur subunit and the molybdopterin-binding subunit contain predicted N-terminal twin-arginine translocation signals, suggesting that the gene products are translocated to the outer membrane after translation. Although there are orthologs of PAE1263 in several *Pyrobaculum* species, orthologs for all three components of the PAE1263-PAE1265 operon were found only in *P. arsenaticum*, the only other archaeon known to grow robustly on arsenate (Table 2) (29). Based on these data, we propose that the PAE1263-PAE1264-PAE1265 operon encodes a membrane-bound ARR.

Few other genes were specifically upregulated by arsenate

(Fig. 2A). These included a putative *arsR* homolog (PAE1592), which may be involved in the regulation of arsenite-responsive genes, and a putative argininosuccinate synthase (PAE2884). Genes annotated as an arsenical pump-driving ATPase (PAE1427) and an arsenite permease (PAE2457) were not upregulated in arsenate-respiring *P. aerophilum* cultures, even when arsenite accumulated to 1 mM (see Table S2B in the supplemental material).

Recovery of a variant *P. calidifontis* strain after growth on arsenate. The *P. calidifontis* genome (Refseq NC_009073) contains orthologs for both PAE1263 and PAE1264 (Pcal_1599 and Pcal_1600) (Table 2). An ORF adjacent to these orthologs (Pcal_1601-trunc; currently annotated as a pseudogene) with strong primary sequence similarity to PAE1265, is truncated after 810 amino acids by a stop codon (TAG) at *P. calidifontis* chromosomal position 1489855 (Fig. 2F). Fourteen of 15 sequencing reads used to assemble the *P. calidifontis* genome in this region unambiguously show a TAG stop at this position, indicating that this was not a sequencing error. However, one read (NCBI trace archive ID number 1617717348) shows a GAG at this position (Glu; the consensus amino acid at the corresponding site in *P. aerophilum* and *P. arsenaticum*), which would produce a 1,170-amino-acid protein with 83% identity to the 1,173-amino-acid PAE1265 protein (Pcal_1601-full) (Fig. 2F). The stop codon at position 1489855 occurs approximately 500 nt upstream of what would be a molybdopterin-binding domain in the full-length Pcal_1601, if translated. Although the *P. calidifontis* culture used for whole-genome sequencing was grown from a single colony, the anomalous sequencing record showing the key GAG Glu codon suggests that this culture may have contained a subpopulation of cells with a variant Pcal_1601 locus encoding a full-length, functional ortholog of PAE1265.

We resequenced the Pcal_1601 locus in our laboratory stocks of *P. calidifontis*, which were directly derived from cultures used for whole-genome sequencing. The PCR amplification of the genomic DNA confirmed the presence of the TAG stop codon, with no evidence for alternative bases at position 1489855. We tested the growth of these *P. calidifontis* cultures with arsenate to determine whether a full-length ortholog of the PAE1265 protein is required for arsenate respiration. *P. calidifontis* grew very slowly and to low densities during the first two passages with arsenate as the only electron acceptor. No growth was observed when arsenate was omitted. Growth during subsequent passages with arsenate was more rapid and produced higher cell densities ($\geq 10^8$ cells/ml). After the sixth passage of *P. calidifontis* on arsenate, we extracted genomic DNA and sequenced the PCR-amplified Pcal_1601 locus again. This revealed a GAG Glu codon at position 1489855. No other differences relative to the reference sequence were observed in the 1-kb PCR amplicon, suggesting that the cultivation of *P. calidifontis* with arsenate as the only electron acceptor selected for a strain with a full-length ortholog of the proposed ARR encoded by PAE1265. We have isolated clonal cultures of each strain from single colonies and confirmed that only the strain with the full-length Pcal_1601 grows on arsenate. We are currently investigating the variant strains to describe these in more detail.

Upregulation of a putative thiosulfate or polysulfide reductase during anoxia. Components of the putative membrane

molybdopterin oxidoreductase encoded by PAE2859, PAE2860, and PAE2861, were specifically upregulated in *P. aerophilum* following the onset of anoxia in cultures lacking an alternative respiratory electron acceptor. These included the second and third time points in cultures that received no added terminal electron acceptor, and the last time point in the cultures that received additions of oxygen (Fig. 2B and E). The putative 91-kDa Mo-containing subunit encoded by PAE2859 contains a twin-arginine translocation signal and a MopB-thiosulfate-R-like domain that is conserved in thiosulfate, polysulfide, and sulfur reductases. The putative 31-kDa membrane subunit encoded by PAE2861 contains a domain that is conserved in membrane subunits of polysulfide reductases. Orthologs of the PAE2859-PAE2860-PAE2861 complex were found in the *P. arsenaticum* and *P. calidifontis* genomes, whereas *P. islandicum* and *T. neutrophilus* were missing the membrane subunit (Table 2). Northern analysis of the molybdopterin-containing subunit PAE2859 showed a single band of approximately 3.8 kb, a size consistent with the cotranscription of these three genes, and possibly a small 3' untranslated region (PAE.i1535) (Fig. 2E).

The domain structure and expression patterns of this operon suggest that it is a respiratory reductase of an electron acceptor such as thiosulfate or polysulfide that is not used by *P. aerophilum* in the presence of oxygen, nitrate, arsenate or ferric iron. *P. aerophilum* is poisoned by the presence of elemental sulfur and grows weakly using thiosulfate as an electron acceptor (29, 65). Growth on polysulfide has not been tested. The basal growth medium contained 11 mM sulfate and no other sulfur compounds aside from those present as trace constituents of the yeast extract used as the carbon and energy source for growth. Physiological studies of *Pyrobaculum* species have shown that their growth with different terminal electron acceptors is optimized at different reducing potentials (21). This observation suggests that reducing potential may directly modulate the regulation of respiratory reductases in *Pyrobaculum* and might explain the upregulation of the PAE2859-PAE2861 molybdopterin oxidoreductase in the absence of its cognate substrate.

Limited upregulation during iron respiration. Cultures shifted to ferric-citrate reduced approximately 3.5 mM Fe(III) to Fe(II) during the time course (see Fig. S1E in the supplemental material). A large number of genes were moderately upregulated in these cultures (see Fig. S4A in the supplemental material). However, we were unable to identify genes that exhibited strong and specific upregulation in response to iron, and positive transcriptional responses to iron (see Fig. S4A in the supplemental material) were generally weaker than the negative responses (see Fig. S5A in the supplemental material). A region containing three potential genes (PAE.i1354, PAE2526, and PAE2527) showed a strong initial response to iron that declined over the time course and opposite trends in cultures amended with oxygen or no electron acceptor (see Fig. S4A in the supplemental material). Other genes upregulated by iron exhibited increasing trends over the time course that were also apparent under other respiratory conditions. These included a putative 40.9-kDa monoheme *c*-type cytochrome (PAE0090), a putative thiosulfate sulfurtransferase (PAE2582), and a number of loci adjacent to each of these (see Fig. S4A in the supplemental material). Genes including a succinate dehydrogenase complex (PAE0716-PAE0725) (see Fig. S4B in the

supplemental material) exhibited positive responses that appeared to be more specific but were also relatively modest.

Previous studies have shown that respiratory ferric reductase activity is regulated and primarily associated with the cytoplasmic cell fraction in *P. aerophilum* and *P. islandicum* (20, 21). Specialized membrane proteins and *c*-type cytochromes are important in bacterial iron respiration but were found to be lacking in iron-respiring *Pyrobaculum* cultures. We were similarly unable to identify a specific oxidoreductase for ferric iron respiration. It is possible that genes for respiratory iron reduction, like the genes in the nitrate reductase operon, were expressed under all conditions in our study with only modest differences between respiratory treatments. Alternatively, the lack of apparent specialization for ferric iron respiration may support the theory that respiratory iron reduction evolved early as a primitive process mediated by a variety of oxidoreductases and electron carriers rather than a single specific complex (47).

Transcriptional repression by iron. A large proportion of genes that were differentially expressed between respiratory conditions at a statistically significant level were downregulated in cultures amended with ferric iron. A number of these have annotations suggesting roles in oxidative stress response or iron uptake and homeostasis. These included genes for superoxide dismutase (PAE0274), Mn-catalase (PAE2696), a putative antioxidant protein (PAE0877), a putative ABC transporter for Fe(III) (PAE2699), and a gene annotated as a bacterioferritin comigratory protein (PAE2732) (see Fig. S5A to S5D in the supplemental material). Genes for putative GTP cyclohydrolases (PAE1588-PAE1589, PAE2984-PAE2986), which are involved in folate and flavin biosynthesis, were also repressed in cultures amended with iron (see Fig. S5A, S5F, and S5G in the supplemental material).

The Mn-catalase (PAE2696) was the top-ranked gene in statistical analyses for differential expression between respiratory conditions (see Table S2C in the supplemental material) and was very strongly downregulated by iron (up to 40-fold compared to cultures induced with oxygen) (see Fig. S5A and S5C in the supplemental material). An examination of the catalase gene and an adjacent cluster of iron-repressed genes (PAE2697-PAE2702) revealed a strikingly conserved palindromic promoter motif (see Fig. S5J in the supplemental material). Three nearly identical versions of this motif are located directly over predicted basal regulatory elements for PAE2696, PAE2699, and PAE2700 (see Fig. S5K and S5L in the supplemental material). A genome-wide search for similar motifs revealed a perfect match in the promoter region for a predicted metal transporter that was upregulated at the end of the time course with nitrate and downregulated by iron (PAE0600) (see Fig. S2C and S5E in the supplemental material). No orthologs for PAE2697, PAE2699, or PAE2700 were found in the other *Pyrobaculum* genomes (Table 2). However, the promoter motif is largely conserved in regions upstream of the *P. arsenaticum* and *P. calidifontis* orthologs for PAE2696 (see Fig. S5L in the supplemental material) and PAE0600 (not shown).

Northern analysis of the putative Fe(III) transporter gene PAE2699 showed a band at approximately 0.8 kb, somewhat larger than the annotated size of 624 nt, and a band at 1.3 kb, consistent with the cotranscription of the downstream putative ABC transporter gene (PAE2697) (see Fig. S5D in the sup-

FIG. 3. Summary schematic showing proposed roles for *P. aerophilum* genes in aerobic respiration and O₂ scavenging (A), denitrification (B), arsenate respiration (C), polysulfide or thiosulfate respiration (D), and iron-mediated transcriptional repression (E).

plemental material). Northern analysis of the predicted metal transporter PAE0600 (see Fig. S5E in the supplemental material) showed a very similar expression pattern, with strong upregulation at the end of the time course with nitrate, when denitrification genes were induced, and downregulation after amendment with iron. Northern analysis also confirmed microarray data indicating that a ferritin-like gene (PAE2701) located between the PAE2696-PAE2700 cluster and another iron-repressed gene, PAE2702, was not downregulated by iron (see Fig. S5H in the supplemental material).

Evidence for an archaeal ferric uptake regulator system.

Iron concentrations in the *P. aerophilum* cultures amended with ferric citrate exceeded the concentration in the basal growth medium by more than 1,000-fold (~9 mM compared to ~7 μM). Iron is a cofactor for numerous enzymes and an essential nutrient for nearly all microorganisms. However, the co-occurrence of high intracellular Fe(II) and O₂ can generate reactive oxygen species, requiring organisms to tightly regulate the uptake and storage of iron in the cell (8). In bacteria, many genes involved in iron homeostasis and oxidative stress are controlled by the ferric uptake regulator (Fur) protein, which binds to DNA and acts as a transcriptional repressor when complexed to Fe(II) (8, 30, 62).

The primary targets of transcriptional repression by bacterial Fur are remarkably similar to those found in this study to be repressed by iron in *P. aerophilum*: genes for the oxidative stress response and for the acquisition, transport, and storage of iron. GTP cyclohydrolases, which were repressed by iron in *P. aerophilum*, are involved in reductive iron assimilation in bacteria and may also be controlled by Fur (14, 68). Bacterial Fur systems are mechanistically complex, with posttranscriptional regulatory roles for small regulatory RNAs and for RNA chaperones that are similar to eukaryotic and archaeal small nuclear ribonucleoproteins (37, 38, 40). Whether similar Fur-regulated systems operate in archaea has not been directly investigated, but proteins with Fur-like domains can be found in more than a dozen archaeal genomes, including all those in the *Pyrobaculum* clade.

The *E. coli* Fur binding site consensus sequence has been alternatively modeled as an FF_nR repeat of GATAAT (where F stands for the forward strand, R for the reverse, and n for any nucleotide) (19) and an FnR repeat of AATGATAAT, which shows good agreement with the consensus sites of *Bacillus subtilis* and *Pseudomonas aeruginosa* (12). The motif identified in the PAE2696-PAE2700 locus shows some consistency with both models. The putative *P. aerophilum* motif consists of the

sequence ATTAAC repeating in the pattern FnnnR, which can be viewed in reverse complement as GTTAAT repeated RnnnF (see Fig. S5J in the supplemental material), remarkably similar to the bacterial GATAAT motif. Whether this motif is the target of the *P. aerophilum* Fur homolog remains to be determined. Fur is autoregulatory in many, but not all, bacteria, repressing its own promoter in the presence of iron (16, 15). However, no match for the PAE2696-PAE2700 promoter motif was found in the promoter region of the *P. aerophilum* Fur homolog (PAE2309), and the transcript was not strongly downregulated after induction with iron (see Table S2B and Fig. S5I in the supplemental material). Nevertheless, similarities with the bacterial Fur system suggest a potentially homologous regulation system in archaea.

A transcriptional map of crenarchaeal respiratory versatility. The results of this study provide a map of gene expression for multiple respiratory pathways in *P. aerophilum* and suggest a number of functional and regulatory predictions for further study. Two cytochrome oxidases were expressed in opposing patterns, suggesting that a SoxB-type complex is for oxygen scavenging and a SoxM-type complex is for aerobic growth (Fig. 3A). The nitrate reductase operon was expressed under all conditions examined. In contrast, genes for nitrite reductase and nitric oxide reductase were coordinately upregulated when nitrite accumulated to ~1 mM and were coexpressed with a putative hemerythrin-containing transcriptional regulator that may control nitric oxide-responsive genes (Fig. 3B). A multicopper oxidase upregulated during nitrate respiration may play a specific role in denitrification, possibly as a nitrous oxide reductase (Fig. 3B). During arsenate respiration, *P. aerophilum* upregulated a molybdopterin oxidoreductase complex (Fig. 3C). A full-length ortholog of this complex appears to be required for the growth of *P. calidifontis* on arsenate, providing support for the proposed role as a crenarchaeal ARR. *P. aerophilum* expressed an alternative molybdopterin oxidoreductase operon, which may encode a thiosulfate or polysulfide reductase, in anoxic cultures lacking an alternative electron acceptor (Fig. 3D). Although a number of loci were moderately upregulated during ferric iron respiration, we were unable to identify genes specific for this process.

P. aerophilum cultures shifted to growth with ~9 mM iron exhibited transcriptional repression at many loci with annotations suggesting roles in iron homeostasis and oxidative stress (Fig. 3E). These responses are similar to those controlled by the ferric uptake regulator in bacteria and suggest either convergent evolution, an ancient global regulatory system, or some combination of the two. Evidence for an ancient Fur system would have interesting evolutionary and paleochemical implications. The bacterial Fur regulatory system apparently evolved to cope with iron scarcity caused by the low solubility of Fe(III) oxides in an oxidizing environment and the toxicity posed by intracellular Fe(II) and free O₂. The presumed divergence of the *Bacteria* and *Archaea* occurred well before the global oxidation of the atmosphere and oceans 2.3 to 2.7 billion years ago, when soluble ferrous iron was plentiful and free O₂ was scarce. However, based on the phylogeny of cytochrome oxidases, it has been proposed that the capacity for aerobic growth evolved in the last universal ancestor of *Bacteria* and *Archaea* prior to the broader oxidation of the biosphere, within localized environments enriched in O₂ (10, 11). A conserved,

vertically transmitted Fur system in the *Archaea* might provide additional support for evolutionary adaptation to oxidizing environments in the last universal ancestor and therefore an early emergence of free O₂.

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